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Modulating flowering time and prevention of pod shatter in oilseed rape

John Chandler[2,4], Laurent Corbesier[1,5], Patrick Spielmann[2,6], Josef Dettendorfer[3], Dietmar Stahl[3], Klaus Apel[2] and Siegbert Melzer[1,2,7,*]

*[1]Department of Life Sciences, University of Liège, B22, Sart Tilman, B-4000 Liège, Belgium; [2]Swiss Federal Institute of Technology, Institute for Plant Sciences, Universitätstr. 2, CH-8092 Zürich, Switzerland; [3]PLANTA GmbH, Grimsehlstrasse 31, D-37555 Einbeck, Germany; [4]Present address: University of Cologne, Institute of Developmental Biology, Gyrhofstrasse 17, D-50923 Köln, Germany; [5]Present address: Max-Planck-Institute for Plant Breeding Research, Carl-von-Linné Weg 10, D-50829 Köln, Germany; [6]Present address: University of Zurich, Institute of Physiology, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland; [7]Present address: Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, Ghent University, Technologiepark 927, B-9052 Ghent, Belgium; *Author for correspondence (e-mail: siegbert.melzer@psb.ugent.be)*

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Abstract

Floral induction is a key developmental switch in plants that leads to the production of flowers, fruits and seeds, which are of paramount importance for human life. To meet the demands of several crop harvests per year, or the growth of crop plants in regions with short vegetation times and for the production of ornamental plants, the timing of the floral transition plays is very important. The discovery of genes that are involved in flowering time control in model plants should allow the modulation of this developmental switch also in plants with economic value. By using a transgenic approach, we showed that a single MADS box gene accelerated flowering and seed ripening in summer rape plants. The *MADSB* transgene also partially substituted for the strict temperature requirements for flowering in winter rape plants. Transgenic winter rape plants expressing the *MADSB* transgene also produced more rigid siliques than wild type winter rape plants, and this prevented precocious seed dispersal.

Introduction

Approximately 40 species of the genus *Brassica* are commercially important varieties for oil seeds, leaf and stem vegetables, condiments and forage production. Often the length of the growth period and environmental conditions are important in matching the cultivar's needs with its climate to optimize yield (Friend 1985). For a timely production of seeds, floral induction is a key developmental switch in *Brassica* species used for oil production. This is especially important for northern countries, in which short growing seasons and an early winter can reduce the amount and quality of the harvest considerably.

Both annual and biennial forms are found among closely related *Brassica* cultivars in which flowering time is triggered either by the length of the daily light period (photoperiodism) or a certain time at lower temperatures (vernalization) or a combination of both (Friend 1985). This dependence on environmental signals is often a limiting factor for the growth of crop plants such as *Brassica* species in different hemispheres and for

breeding programmes that require simultaneous flowering of the parental lines.

The genetic basis of floral induction has been analyzed by using the model plant *Arabidopsis thaliana*, a facultative long-day and facultative cold-requiring plant of the *Brassicaceae* family. By mutational analyses, many genes have been identified that regulate flowering time through different pathways, including photoperiod- and vernalization-dependent pathways (Koornneef et al. 1998; Simpson and Dean 2002; Mouradov et al. 2002). Constitutive expression of several of these genes modulates flowering time in transgenic *Arabidopsis* plants (Weigel and Nielson 1995; Simon et al. 1996; Kania et al. 1997). However, it is not known whether the results derived from *Arabidopsis* can be easily generalized to control flowering time in other plants, crop plants in particular.

The constitutive expression of flowering time genes might be a feasible approach to overcome environmental constraints for floral induction. MADS box genes encode a large family of transcription factors, many of which are involved in floral organogenesis, and it has been shown that some also regulate flowering time (Ng and Yanofsky 2001). The MADS box gene *MADSB* in mustard (*Sinapis alba*) and the close homologue *FRUITFULL* (*FUL*) in *Arabidopsis* are up regulated in apical meristems very early during the transition to flowering (Menzel et al. 1996; Mandel and Yanofsky 1995). The *Arabidopsis ful1* mutant is delayed in flowering in continuous light (Ferrándiz et al. 2000a), and shows an even greater delay in flowering time under short day conditions (Melzer, unpublished). A combination of the *ful* mutation with *apetala1* (*ap1*) and *cauliflower* (*cal*) mutations shows a striking non-flowering phenotype of the triple mutant (Ferrándiz et al. 2000a). Therefore, the *MADSB*/*FUL* gene is a good candidate gene for controlling flowering time in crop plants via transgenic approaches.

The most obvious phenotype of *ful* mutants is a defect in carpel and fruit development, leading to short fruits with crowded seeds (Ferrándiz et al. 2000a). In addition, it has been shown that *FUL* negatively regulates two other MADS box genes, *SHATTERPROOF1* and *2 SHP1* and *2*) that are closely related and exhibit an overlapping expression pattern to each other. Single mutants of both genes show a wild type phenotype, but the *shp1shp2* double mutant has fruits which do not open, indicating that both genes act together to control seed dehiscence in *Arabidopsis* (Liljegren et al. 2000; Ferrándiz et al. 2000b).

In order to modulate flowering time in crop plants, we have over-expressed several flowering time genes in annual as well as in biennial *Brassica napus* plants. Here we report that the 35S:*MADSB* transgene from mustard had an effect on flowering time in transgenic rape plants and that transgenic winter rape had a lowered vernalization requirement. In addition, the over-expression of *MADSB* caused an altered carpel development that prevented seed loss in winter rape plants.

Materials and methods

Plant material and growth conditions

Drakkar summer rape and Erox winter rape transgenic lines and the corresponding wild type control plants were grown either in growth chambers under long-day conditions (16 h light/8 h dark) at 20 °C under fluorescent tubes emitting a photon flux density of 150 μmol m^{-2} s^{-1} or in greenhouses. Flowering time was assessed as the time until the opening of the first flower. The winter rape was vernalized 1 week after sowing for 50 days at 4 °C at low light intensities under long-day conditions in a growth chamber or at 11 °C for 34 days during the winter in a greenhouse.

Over-expression of MADSB in oilseed rape

To over-express *MADSB* under the control of the 35S CaMV promoter in rape plants, an *Eco*RI fragment containing the complete *MADSB* open reading frame from mustard (Menzel et al. 1996) was inserted into the pRT104 vector (Töpfer et al. 1987) and transformed into *E. coli* DH5α cells. After partial digestion with *Hind*III, a fragment containing the expression cassette was isolated and inserted into the BIN19 vector (Bevan 1984) and the recombinant vector pBIN19-*MADSB* was transformed into *Agrobacterium tumefaciens* strain C58C1. The recombinant *Agrobacteria* were used to transform summer and winter rape lines by a standard procedure (Moloney et al. 1989).

Regenerating plants were selected on agar plates containing MS media supplemented with 50 mg/l kanamycin. Seeds from T2 and T3 plants were tested for homozygosity on kanamycin plates and T3 and T4 homozygous lines were used for the experiments. From 15 transgenic plants of the summer rape cultivar Drakkar, 10 lines were analyzed in greater detail (DB1-DB10). For the winter rape cultivar Erox, seven independent transformed lines were obtained and three were analyzed in more detail (EB1-EB3).

Light microscopy

Carpels from summer and winter rape wild type plants and transgenic lines were embedded in Technovit 7100 plastic resin (Kulzer) and cut with a rotary microtome. Transverse sections of 2–3 μm were stained for 5 min in 0.01% (w/v) aqueous toluidine blue. The sections were rinsed with water and analyzed either with a Zeiss Axiophot or a Leitz Diaplan microscope.

RNA gel blot analyses

For RNA gel blots, RNA was extracted from 3-week-old seedlings with guanidinium isothiocyanate, as described (Melzer et al. 1990). Fifteen micrograms of total RNA were separated on a 1% agarose gel containing 1% formaldehyde and transferred onto Nylon PALL Biodyne B membranes. Ethidium bromide staining was used as a control for RNA loading. The hybridization probe, without the MADS-box coding region, was amplified by PCR as previously described (Menzel et al. 1996).

Results

Regulation of flowering time in oilseed rape by constitutive expression of MADSB

The level of transgene expression in the different summer rape lines varied in 3-week-old seedlings from undetectable in line DB2, to very weak in line DB5, to intermediate in most of the lines and a very high expression in lines DB9 and DB1. Expression of the orthologous *MADSB* gene from *Brassica* was not detectable with the mustard probe (Figure 1). The constitutive expression of

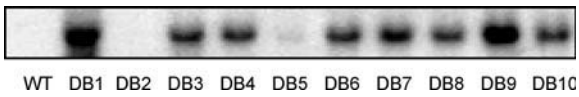

Figure 1. Transgene expression in Drakkar summer rape plants. The northern blot shows a comparison of 35S:*MADSB* transgene expression in wild type summer rape plants and the transgenic summer rape lines DB1 to DB10.

MADSB caused significant earlier flowering in most transgenic summer rape lines compared with corresponding wild type plants (Figures 2a and 3). Only line DB2, which had no detectable transgene expression levels, flowered at the same time as wild type plants. The shortening of the vegetative phase was between 3 and 15 days and no pleiotropic effects on floral development and seed yield were observed. This flowering time phenotype was also observed in the next generation of all transgenic lines.

In order to study whether the strict vernalization requirement of winter rape plants can be circulated by over-expression of *MADSB*, we compared three transgenic lines with wild type plants after a weak vernalization treatment at 11 °C for 34 days at the rosette stage during winter time in a greenhouse. Wild type plants flowered 83 days after the end of the vernalization treatment. The transgenic line EB1 flowered at the same time as wild type plants, whereas line EB2 flowered after 60 days and line EB1 flowered 53 days after the vernalization treatment (Table 1, Figure 2b). None of the lines flowered without any vernalization. By using a more strict vernalization treatment at 4 °C for 50 days, plants of the earliest flowering line EB3 reached anthesis about 10 days earlier than the corresponding wild type winter rape plants. A comparison of the results from the two different vernalization treatments indicates that the transgenic plants are either more responsive to vernalization, which is only visible at milder vernalization treatments, or have a lower vernalization requirement than the corresponding wild type winter rape plants.

Constitutive MADSB expression leads to the formation of indehiscent siliques

In a typical *Brassica* fruit, two carpels enclose two locules, which are separated by a central

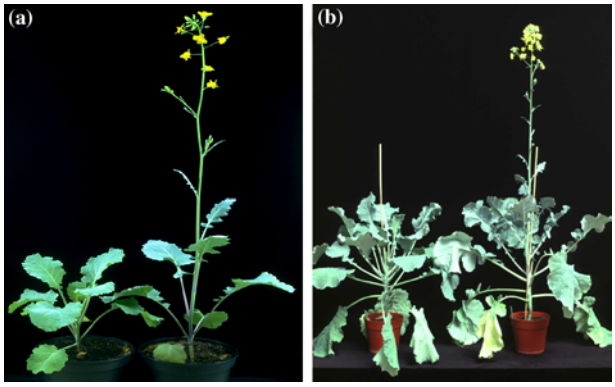

Figure 2. Modulation of flowering time in rape plants by constitutive expression of *MADSB*. (a) Transgenic 35S:*MADSB* lines of the Drakkar summer rape cultivar (right) flowered up to two weeks earlier under greenhouse conditions than the corresponding Drakkar wild type summer rape plants (left) without any effect on the development of flowers or seed set. (b) Transgenic 35S:*MADSB* plants of the Erox winter rape cultivar (right) flowered earlier after vernalization treatments than the corresponding Erox wild type winter rape plants (left).

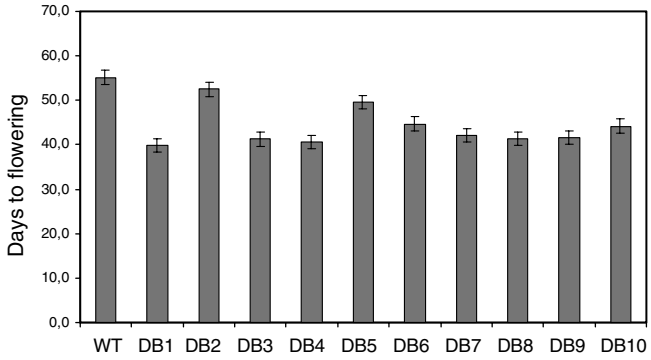

Figure 3. Modulation of flowering time by 35S:*MADSB*. (a) The graph shows a comparison of 10 selected transgenic Drakkar summer rape lines with wild type Drakkar plants. The transgenic summer rape lines DB1 to DB10 flowered between 3 and 15 days earlier than the corresponding wild type summer rape.

Table 1. Days to flowering after vernalization treatments in wild type and transgenic Erox winter rape plants

Genotype	50 days 4 °C	34 days 11 °C
Erox	53.1 ± 2.5	83.3 ± 3.5
EB1	n.a.	84.3 ± 2.8
EB2	n.a.	60.3 ± 2.6
EB3	43.8 ± 3.2	53.4 ± 3.6

The data are presented as the mean ± SD of six or seven individual plants per genotype, n.a. = not analyzed.

pseudoseptum. The fruit opens after ripening at a dehiscence zone that develops along the connective tissue at the boundary of the two carpels and the pseudoseptum over the entire length of the silique (Spence et al. 1996). During wild type fruit ripening, the cells of the dehiscence zone became lignified, allowing the two valves to separate from the replum after maturation of the fruit (Figure 4a and b). In both winter and summer rape plants, the constitutive expression of the *MADSB* gene caused a modified dehiscence zone (Figure 4c–f). In the carpels of transgenic winter rape plants, no dehiscence zone with lignified cells was formed (Figure 4c). The sharp incision at the fusion zone of the two valves at the replum of a wild type silique (Figure 4b) was completely filled with cells, showing no valve–replum margin (Figure 4c). While the alteration of the dehiscence zone did not alter seed viability or yield, siliques developed that remained closed after ripening, which prevented seed loss prior to or during silique harvest (Figure 5a and b).

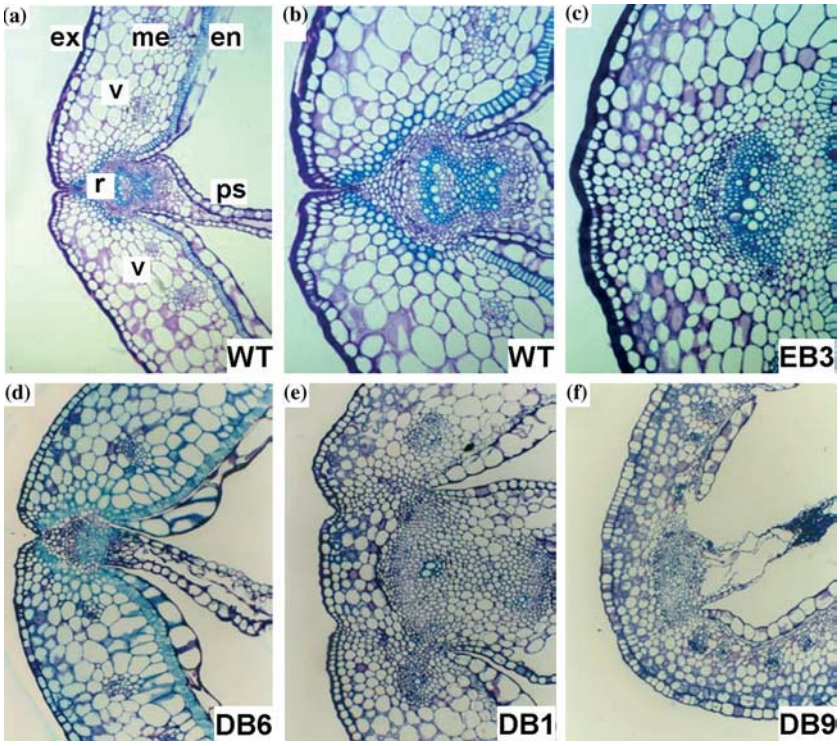

Figure 4. Effect of the 35S:*MADSB* transgene expression on the morphology of siliques in rape plants. Transverse sections of siliques from a wild type Erox winter rape plant (a, b), the transgenic Erox winter rape line EB3 (c), as well as the transgenic Drakkar summer rape lines DB6 (d), DB1 (e) and DB9 (f), which show a gradual alteration of the dehiscence zone are shown. (b) shows the same transverse section of a silique as in a, but at a higher magnification. Lignified cells of the dehiscence zone as well as those from the vascular bundles and from the inner cell layer of the endocarp were visualized by toluidine blue staining, which leads to an intense blue staining of the lignified cell walls. v = valve, r = replum, ex = exocarp, me = mesocarp, en = endocarp, ps = pseudoseptum.

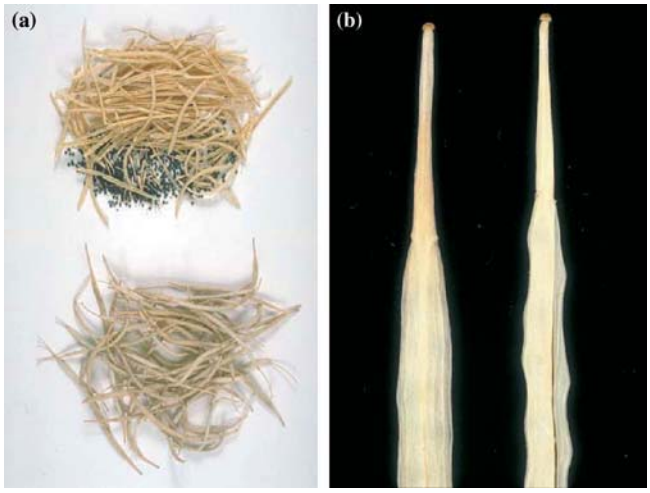

Figure 5. Effect of 35S:*MADSB* transgene expression on fruit dehiscence in Erox winter rape plants. Siliques of Erox wild type plants (a (top), b (right)) and of the transgenic Erox line EB3 (a (bottom), b (left)) shortly after the harvest. The siliques of the wild type winter rape plants are open at the valve–replum boundary while those of the transgenic winter rape line are still closed (b), which leads to seed dispersal from wild type siliques (a).

In the transgenic summer rape lines, we also observed alterations in silique structure comparable to those in the winter rape lines. In a transgenic line with a low transgene expression (DB6), the incision between the carpels and the lignified cells of the dehiscence zone was still visible. The incision disappeared gradually with an increase of transgene expression and was filled except for two small incisions or was completely filled in the highest expressing lines DB1 and DB9, respectively (Figure 4d–f). In addition, no lignified cells were visible in the dehiscence zone in DB1 and DB9 siliques. Despite the alterations in the dehiscence zone, low mechanical force, which is already imposed by a manual harvest of the siliques, was sufficient to open the siliques of all transgenic summer rape lines as well as those of the wild type plants. The winter rape lines with a similar expression of the transgene (data not shown) exhibited a tight closure of the siliques during harvest (Figure 5a and b).

Discussion

In recent years, much knowledge has accumulated from model plants such as *Arabidopsis* and rice on how flowering is controlled by environmental conditions (Searle and Coupland 2004). However, these data have not been applied to a large extent to modulate flowering time in crop plants. That over-expression of the *MADSB* gene caused early flowering in summer rape plants is in agreement with previous results obtained in mustard and *Arabidopsis* showing an up regulation of *MADSB* and *FUL* in apical meristems very early during the floral transition (Mandel and Yanofsky 1995; Menzel et al. 1996) and with those showing that the *Arabidopsis ful1* mutant was late flowering, whereas the constitutive expression of *FUL* under the control of the 35S promoter dramatically reduced the time to flowering in *Arabidopsis* (Ferrándiz et al. 2000a). It has also been shown that the expression of the *MADSA*/*SOC1*/*AGL20* orthologous genes in different *Brassicaceae* species is correlated with flowering time as it is in mustard and *Arabidopsis* (Kim et al. 2003). This suggests that rape plants may share common floral induction pathways with *Arabidopsis* and that genes which have been shown to be involved in flowering time control in *Arabidopsis* also have the same functions in other *Brassicaceae* species.

In several late flowering mutants and accessions of *Arabidopsis*, a vernalization response is dependent on the down-regulation of the MADS box gene *FLOWERING LOCUS C* (*FLC*). During cold treatment, *FLC* transcript levels decrease, allowing flowering to occur (Michaels and Amasino 2000). In *Brassica*, two vernalization-dependent *Brassica* QTLs show colinearity to the *Arabidopsis* genome where *FLC* and *FRI*, which promote *FLC* expression, are located (Osborn et al. 1997). Vernalization also dramatically reduced the level of *BnFLC* transcripts in winter rape, causing the cold treated plants to flower, while the constitutive expression of an *Arabidopsis FLC* transgene in an early flowering cultivar of *B. napus* caused a delay in flowering by 2–6 weeks (Tadege et al. 2001). Since *Arabidopsis flc* null mutants still responded to vernalization, it has been suggested that other genes might also be involved in the vernalization response. Recently, a paralogue of *FLC*, *MAF2*, has been identified in *Arabidopsis*, which also acts as a floral repressor and is down regulated by vernalization, although later and in an independent manner from *FLC* (Ratcliffe et al. 2003). It was shown, in comparison to wild type plants, that *maf2* mutants responded to short vernalization treatments, indicating that *MAF2* is involved in preventing vernalization effects by short cold periods (Ratcliffe et al. 2003). Therefore, our observation that the *MADSB* transgenic lines flowered earlier after a short vernalization treatment, but did not flower without vernalization, suggests that the over-expression of *MADSB* in winter rape plants might circumvent repression of short vernalization responses via a *MAF2* homologue and not through *FLC*. To understand further which gene functions are limiting to overcome the vernalization requirement of winter rape, additional studies are certainly necessary. However, since the *MADSB* transgene did not cause any other pleiotropic effects, such as infertility or lower seed set as observed by the use of other MADS box transgenes (M. D'Aloia and S. Melzer unpublished), it could be a very useful candidate gene for modulating flowering time in crop plants.

In *Arabidopsis*, *FUL* negatively regulates the *SHP1* and *SHP2* genes, which are known to regulate seed dispersal (Ferrándiz et al. 2000b; Liljegren et al. 2000). Similarly to the transgenic

rape lines in this study, 35S:*FUL Arabidopsis* plants showed a decreased lignification of the cells adjacent to those where the dehiscence zone normally forms, leading to the formation of indehiscent fruits that failed to disperse their seeds normally (Ferrándiz et al. 2000b). An identical phenotype was observed by over-expressing the *DEFH28* gene, a putative *FUL* orthologue from *Antirrhinum*, in *Arabidopsis* (Müller et al. 2001), and expected to see the same phenotype in transgenic rape plants over-expressing *MADSB*. We did indeed observe alterations in silique structure in transgenic rape plants that were similar to those in *Arabidopsis shp1shp2*double mutants and *FUL* over-expressing plants, suggesting that the rape homologue of *MADSB* and *FUL* plays a similar role during fruit development. In addition, we observed that *MADSB* over-expression in tobacco, in which capsules and not siliques are formed, also caused a non-opening phenotype of the capsules in transgenic tobacco (P. Smykal, R. Gleissner and S. Melzer, unpublished), indicating that the molecular mechanisms of carpel opening are conserved in different species with different carpel opening mechanisms.

The siliques of the transgenic winter rape lines remained tightly closed during dehydration, which prevented seed loss after silique maturation and during harvest, which can reach annual yield losses of 20–50% in wild type oilseed rape plants (Roberts et al. 2002). However, it remains unclear why the transgenic summer rape lines did not exhibit a non-opening silique phenotype, even though the structure of the dehiscence zone was similarly altered as in transgenic winter rape lines. Recently, it has been shown that REPLUMLESS (RPL), a homeodomain protein, also plays a role in the formation of replum-valve margin identity in *Arabidopsis* (Roeder et al. 2003). It has also been demonstrated that RPL negatively regulates the *SHP* genes. Therefore, both FUL and RPL are required to limit the expression of the *SHP* genes to develop the valve margin precisely at the valve–replum boundary. The *ALCATRAZ* (*ALC*) gene is related to the myc/bHLH family of transcription factors and promotes the differentiation of a strip of non-lignified cells between layers of lignified cells at the valve margins. In *alc* mutants, the separation of the valves from the replum is blocked, indicating that in addition to lignification, the presence of non-lignified cells at the boundary of the valves also plays an important role in fruit dehiscence (Rajani and Sundaresan 2001). In addition to downstream target genes that encode cell wall degrading enzymes such as cellulases and pectinases (Ferrándiz 2002), there could also be other, currently unidentified regulatory genes involved in establishing the formation of the dehiscence zone. This suggests that subtle differences might exist between rape cultivars in the co-ordination of signaling events involved in fruit dehiscence, which did not allow the prevention of pod, shatter in the summer rape lines by *MADSB* over-expression.

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